PCR Based Secondary Screening of Cloned DNA Used in Sequencing Decisions

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Introduction

Sequencing cloned DNA in order to find regions of interest can be one of the more expensive and/or time consuming aspects of microsatellite development. Many times sequences obtained contain either no region of interest or an initially useless sequence due to nearness of repeats to one end, making primer design impossible. In an effort to shorten development time and reduce waste of funds a second screening protocol, following blue-white screening and purification of promising plasmids, was developed and successfully employed. Using the additional information success at obtaining initially valuable sequence information reached 100%.

Methods

Following extraction of pUC19 plasmids from *E. coli* cultures two polymerase chain reactions (PCR) were run. One PCR was performed using the same primers used by the sequencing facility, where they were used to sequence first forward (21M13 = 5'-TGTAAAACGACGGCCAGT-3') and then reverse (Reverse 2 = 5'-TCACACAGGAAACAGCTATGAC-3'), and a second PCR was performed using the "sequencing primers" along with a primer pool consisting of microsatellite repeats being screened for, which had been used previously in blue/white colony screening [$(TC)_{10}$, $(TG)_{10}$, $(CAC)_5CA$, $CT(CCT)_5$, $(TGTA)_6TG$, $CT(ATCT)_6$]. Pairs of PCR products were then run on 16 cm 8% polyacrylamide gels.

Results / Interpretation

- Where primers bind to produce observed bands

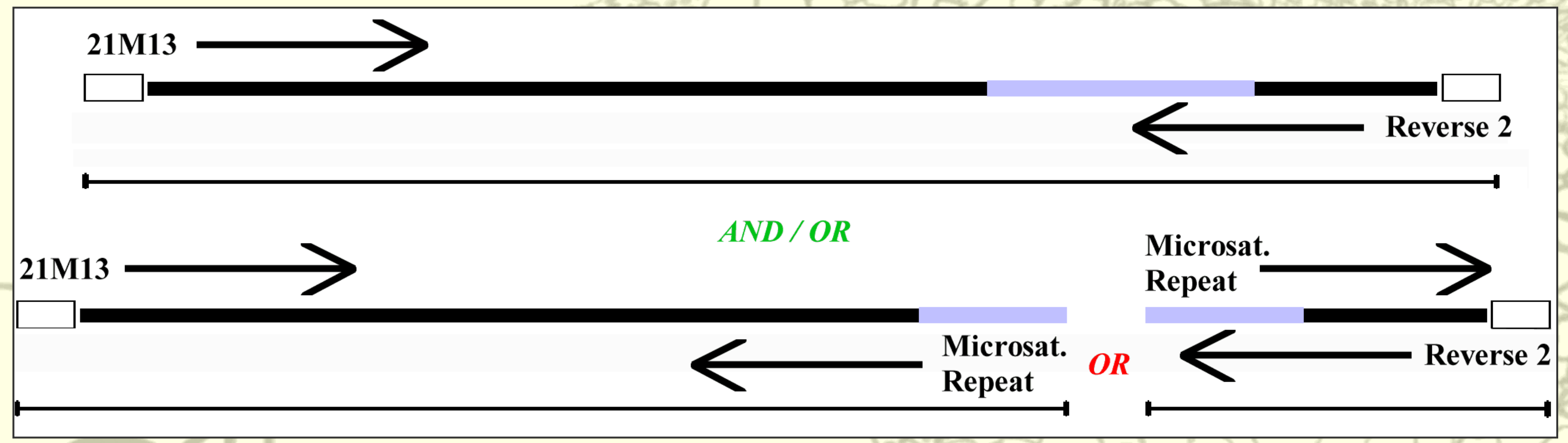

Figure 1. Locations of primer binding sites are indicated and fragment length is represented by the bar beneath. Internal light blue area represents a region of DNA containing microsatellite repeat units. Sequences used in microsatellite primer pool ensured only one of the short products was possible.

"Sequencing primers" (21M13 and Reverse 2) bind at the boxed points (Figure 1) and produce a fragment that represents the entire length of the DNA that was inserted into the circular plasmid prior to being transferred into *E. coli* for plasmid copy multiplication. However, if a primer binds at a point within the inserted DNA there is potential for production of a shorter fragment as well. These possible alternate (or additional) smaller products are represented by the shorter fragments in the figure that have a microsatellite repeat as an end point.

- Typical PCR product band patterns observed

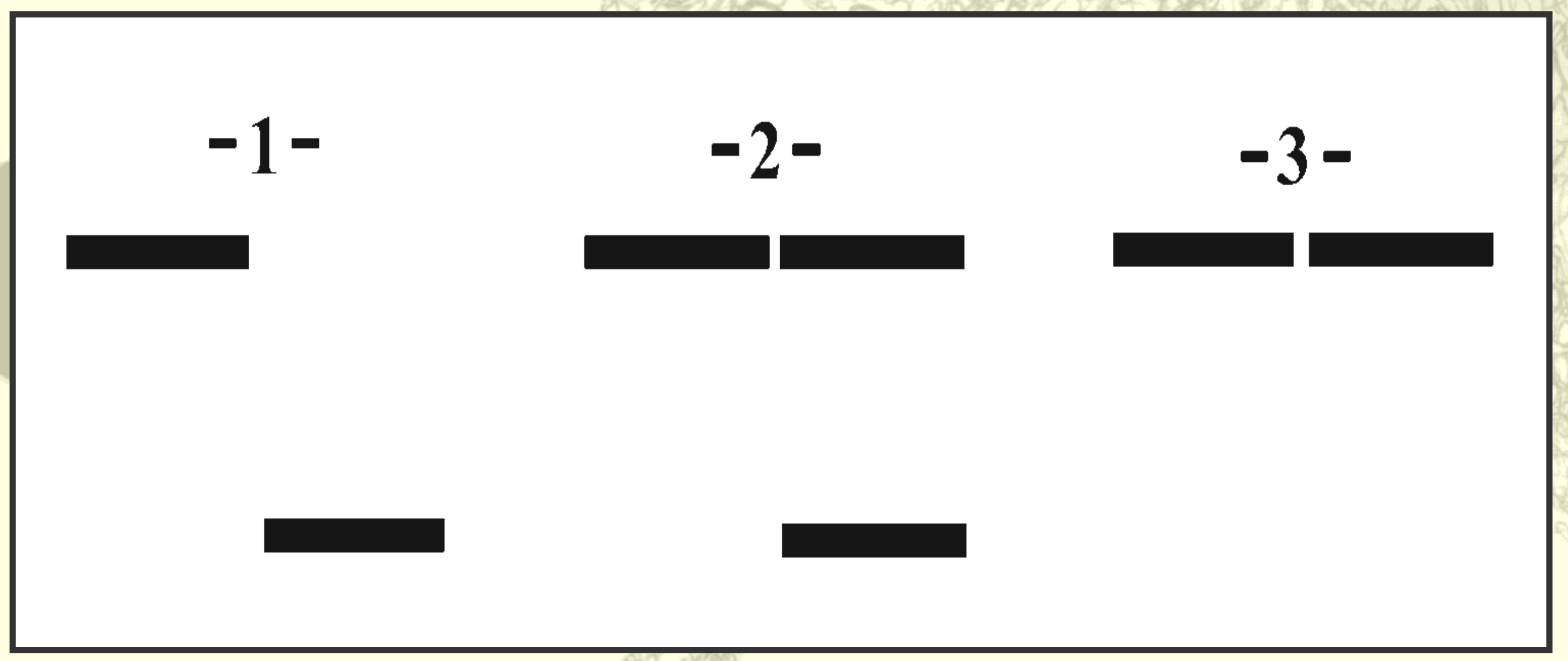

Figure 2. Three patterns are generally visible in the side-by-side PCR product profiles where "screening oligos" were used as primers and compared to the "sequencing primer" PCR products that represent the entire length of the inserted DNA: (1) Only a shorter length fragment is produced in comparison to the entire inserted DNA fragment length. (2) Both a shorter fragment and as well as a fragment the same length as the original inserted DNA are visible. (3) Only a fragment the same length as the original total DNA insert is produced and no shorter fragment is visible.

In the first case (1) it appears that the "screening oligos" (which are simply synthesized sequences of repeated DNA) compete strongly with the "sequencing primers" so that the primary product is a shorter fragment that indicates the distance a set of microsatellite repeat units matching one of the "screening oligos" is from one edge of the inserted DNA sequence.

Competition for primer binding appears less pronounced when both original length and shorter fragments are produced (2). However, in the last example (3) the "screening oligos" do not appear to bind at all, so no shorter fragment is produced and probability of the DNA insert containing a microsatellite repeat is low.

- Photographic example of band patterns observed

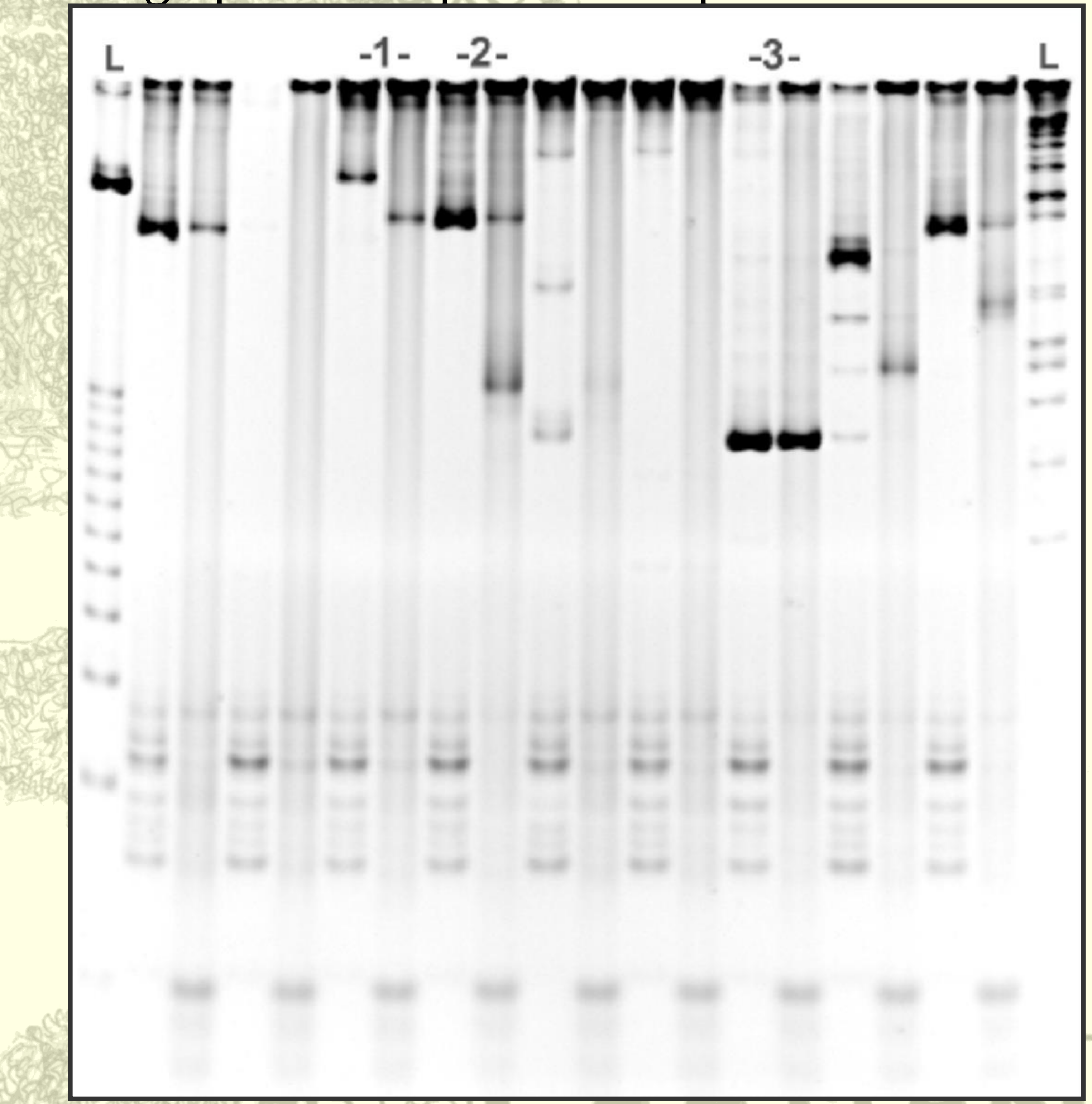

Figure 3. Numbers above paired lanes match the types of products identified in Figure 2 and its accompanying description. "L" indicates the molecular weight ladders used to determine fragment lengths. The short bands near the bottom are produced by the primers interacting among themselves during PCR.

Conclusion

Using this procedure we were able to maximize sequencing time, minimize expense, and found microsatellite repeats ready to have primers designed for them in all sequence data. The benefit of knowing repeat units were deep enough within the DNA insert to allow design of primers was particularly useful. Previously about 50% of sequences did not contain sufficient repeat length to warrant primer design and around 10% had repeats located too close to one end of the inserted DNA to enable good primer design.